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COMPETING FINANCIAL INTERESTS

P.A. and M.P.C. have a patent application on the technology described here. The other authors do not have competing financial interests.

Abstract

Background: Despite the developmental impact of chromosome segregation errors, we lack the tools to assess environmental effects on the integrity of the germline in animals.

Objectives: Here, we report the development of an assay in *C. elegans* that fluorescently marks aneuploid embryos following chemical exposure.

Methods: We qualified the predictive value of the assay against chemotherapeutic agents as well as environmental compounds from the ToxCast Phase I library by comparing results from the *C. elegans* assay with the comprehensive mammalian *in vivo* endpoint data from the ToxRef database.

Results: The assay was highly predictive of mammalian reproductive toxicities with a 69% maximum balanced accuracy. Finally, we confirmed the effect of select compounds on germline integrity by monitoring germline apoptosis and meiotic progression.

Conclusions: We provide here a comprehensive strategy for the assessment of environmental effects on germline function.

Introduction

Aneuploidy originates from chromosome segregation errors during the two highly regulated programs of cell division: mitosis and meiosis. Meiosis differs significantly from mitosis as it reduces the number of chromosomes in half to produce haploid gametes: the egg and sperm. Human female meiosis, in particular, is inherently prone to errors, as evidenced by the high incidence and complexity of aneuploidies in stillbirths and spontaneous abortions (Hassold and Hunt 2001). While the etiology of aneuploidy is incompletely understood, evidence from mammalian studies suggests that exposure to diverse chemicals including chemotherapeutic agents, alcohol, plastics and pesticides, may be causative (Hales et al. 2005; Harkonen 2005; Hunt et al. 2003). However, despite the relevance of meiotic aneuploidies for reproductive health, we are currently unable to *efficiently* and *comprehensively* interrogate the multitude of chemicals in the environment for their effect on germline function and reproductive health.

Several programs at the NIH, FDA and EPA in the United States have identified a critical need in chemical risk assessment and initiated large-scale research programs (ToxCast, Tox21) using high-throughput screening (HTS) assays for predictive toxicology (Dix et al. 2007; Kavlock et al. 2012; Krewski et al. 2010). In line with these efforts, we have developed an HTS platform for environmental toxicants based on germline dysfunction in the roundworm *Caenorhabditis elegans*. *C. elegans* offers significant advantages for this purpose: (1) a high degree of conservation of key mammalian meiotic pathways, (2) a well-studied model system for the study of meiosis and (3) a vast array of cytological, genetic and biochemical tools available (Colaiacovo 2006). Of particular interest was using *C. elegans* in a novel first-tier HTS strategy that would detect abnormal chromosome numbers. We chose to focus on environmental disruption of female meiosis since mammalian oogenesis encompasses events from early

embryonic stages to adulthood, and is therefore especially difficult to study. Here, we report the development of a platform that rapidly and comprehensively interrogates the landscape of environmental chemicals for potential effects on germline function, induction of aneuploidy and prediction of mammalian reproductive deficits.

Methods

C. elegans genetics and growth conditions

C. elegans strains were cultured according to (Brenner 1974) at 20°C on Nematode Growth Medium plates. The N2 Bristol strain was used as wild-type strain. The following mutations and chromosome rearrangements were used in this study: LGIV,*col-121(nx3), him-8*(*e1489*); LGV, yIs34[*Pxol-1::GFP*, *rol-6*], bcIs39[*Plim-7::ced-1::GFP, lin-15*].

Drug treatments and screening procedure

All chemicals were purchased from Sigma-Aldrich, Saint-Louis, MO and dissolved in DMSO (0.1M). Chemicals not dissolvable in DMSO at 0.1M were not considered for this screen. Final DMSO and chemical concentrations were 0.1% and 100µM respectively, except for Mancozeb, Dicofol, 2-(Thiocyanomethylthio) benzothiazole (TCMTB), Phosalone, Chlorophene, Endosulfan, Parathion-methyl, further diluted 10-fold to circumvent lethality. Chlorpyrifos-methyl was used at 1µM for the same reason.

Worms were synchronized by sodium hypochloride treatment of an adult population to generate age-matched embryos (Stiernagle 2006). The embryos were cultured on eight 10cm NGM plates seeded with bacteria for 3 days at 20ºC to generate a large pool of L4-stage worms that was resuspended in M9 buffer with bacteria. Live bacteria were used as described in numerous other

chemical screens in *C. elegans* [see (Boyd et al. 2010a; Boyd et al. 2010b)] which, considering the screen's relatively low false positive and negative rates, is not likely to be detrimental to this assay. After quantification under the microscope of the number of worms in population samples, 300 worms were dispensed in each well of a 24-well plate to which the chemicals were subsequently added. Each plate contained a negative control (0.1% DMSO) as well as a positive control (100μM nocodazole). The worms were then incubated with shaking for either 24hr or 65hr at 25ºC. Following this incubation, worms were transferred to 1.5ml tubes, settled by gravity and washed in M9 before being transferred to a slide and mounted with a coverslip for assessment of GFP-positive embryos under an upright fluorescent microscope (Leica, Buffalo Grove, IL).

All statistical analyses following the *C. elegans* screen were performed using the two-tailed Mann-Whitney test with a 95% confidence interval unless specified otherwise.

Predictivity analysis

To assess the predictive value of the *C. elegans* screen against mammalian *in vivo* reproductive toxicity data, ToxRefDB (Martin et al. 2009) endpoints indicative of decreased female fertility were dichotomized with respect to their lowest effect level in a multigenerational study (MG-LEL). There were 47 compounds with multigenerational reproductive toxicity study data. Those compounds with a MG-LEL≤ 500 mg/kg/day were considered positive reproductive toxicants (n=20), while those with available multigenerational study data and no MG-LEL in that range were considered negatives (n=27). There was a subset of 7 compounds that did not have associated ToxRefDB data; these were excluded from this portion of the analysis. The fold-change cutoff criteria for a positive hit in the *C. elegans* assay was iteratively increased from the

lowest observed value in the assay to the highest, and sensitivity (true positive rate), specificity (true negative rate) and balanced accuracy (BA, the average of sensitivity and specificity) were calculated for each cutoff value. A similar procedure was followed for each individual multigenerational endpoint (with >2 positive compounds) based on iteratively increasing the cutoff value in the *C. elegans* assay at each time point and calculating the relative risk. Statistical analysis was performed using Rv2.13.0 (code included in Supplemental Material).

Embryonic viability measurement

Embryonic viability was performed three times for each exposure as described in (Allard and Colaiacovo 2010). Briefly, the number of eggs laid and of hatched larvae was recorded following a 24hr exposure to DMSO and nocodazole.

Apoptosis assay and germline nuclear analysis

Quantitative analysis of germ cell apoptosis was performed using the *Plim-7::ced-1::GFP* strain as described in (Saito et al. 2009). High-resolution images of germline defects were captured and processed as described in (Allard and Colaiacovo, 2010).

Automated fluorescence reading

A COPAS BIOSORT (Union Biometrica, Holliston, MA) was used for automated worm reading and sorting. Briefly, following a 24hr exposure, the worms were washed at least 3 times in M9 buffer. N2 wild type strain was compared to *him8, Pxol-1::GFP* to ascertain the presence of GFP-positive embryos and adjust reading settings accordingly. The reading parameters used were time-of-flight (ToF) for the X-axis and GFP peak height for the Y-axis. The number of events per sample was 5,000 except for the *him-8* analysis where 1,000 events were read. A non-gated mixed population was used (mainly adults and embryos) from which only the objects of a

size consistent with embryos were analyzed. The threshold to determine debris and GFP-negative embryos versus positives was set using a control population of untreated WT worms.

Results

Establishing a chemical screen for embryonic aneuploidy

The strategy takes advantage of the rare proportion of male progeny (XO, <0.2%) that naturally arises in wild type hermaphroditic (XX) populations, due to meiotic segregation error of the X-chromosome (Hodgkin et al. 1979). As disruption of meiosis very frequently leads to increased non-disjunction and aneuploidy, it correlates with a 'High Incidence of Males' phenotype (Him) due to errors in X chromosome segregation. This phenotype is also accompanied by an elevated embryonic lethality that follows from errors in autosomal chromosome segregation (Dernburg et al. 1998; Hodgkin et al. 1979). To easily detect male embryos *in utero* and circumvent embryonic lethality, a male specific promoter (*xol-1*) is used to drive expression of GFP. This allows quick identification of male embryos by the appearance of "green eggs" within the worm's hermaphrodite uterus. The *Pxol-1::gfp* transcriptional reporter strain has been used in the context of a genetic screen, named the "Green eggs and Him" screen, which led to the isolation of an allele of the meiotic recombination factor, *msh-5* (Kelly et al. 2000; Nicoll et al. 1997).

We developed a chemical strategy using the *Pxol-1::gfp* strain (Figure 1). Specifically, liquid cultures of the strain are exposed to chemicals of interest at 100μM, a concentration commonly used in chemical screens in *C. elegans* (Boyd et al. 2010a; Boyd et al. 2010b). The worm germline consists of nuclei simultaneously moving from the distal to the proximal end of the gonad and progressing through the meiotic stages in a synchronous manner. This establishes a spatial and temporal gradient of meiotic progression in *C. elegans* with well-characterized timing

of events (Jaramillo-Lambert et al. 2007; Pazdernik and Schedl 2013). Consequently, we exposed the worms for durations of 24hr and 65hr to capture the effects of exposure at distinct stages of germline progression. Aneuploidies generated after a 24hr test interval arise from the impairment of late meiotic (late pachytene and onwards) and early embryonic processes, whereas the 65hr interval captures aneuploidies originating from the disruption of any mitotic and meiotic events in the germline in addition to early embryonic stages. Following exposure, the worms were readily observed under a fluorescence microscope. The number of GFP-positive embryos were counted and normalized to the total number of embryos present to correct for decreased embryo production. We also established the automated detection and sorting of the GFP-positive worms by using the COPAS biosorter for sorting of viable worms and embryos. The use of a flow cytometry sorting system allows us to scale up the numbers of chemicals being tested and the speed of screening, thus enabling high-throughput capability (see below).

To discriminate between germline and embryonic chemically-induced defects, we followed the fluorescence screen with two assays: (1) a reporter-based germline apoptosis assay (Zhou et al. 2001), and (2) DAPI-staining of the germline nuclei. These two complementary tests respectively measure induction of the meiotic DNA damage checkpoint (Gartner et al. 2008) and identify the nature of the germline nuclear defects responsible for apoptotic induction and the generation of aneuploidy.

Chemical induction of aneuploidy in *C. elegans* and determination of aneugenic potency

Induction of aneuploidy in *C. elegans* has, to our knowledge, never been described in a chemical screening approach. To verify that *Pxol-1::gfp* reports chemical induction of aneuploidy, we

tested exposure of these worms to the microtubule disruptor nocodazole. We expected nocodazole to promote chromosome segregation errors during the germline mitotic and late meiotic stages as well as during early embryonic stages (Kitagawa and Rose 1999; Stear and Roth 2004). Thus, nocodazole should induce a high number of GFP-positive embryos corresponding to increased X-chromosome missegregation. Indeed, worms exposed to 100µM nocodazole for either 24 or 65hr showed a statistically significant increase in the number of GFP-positive embryos compared to DMSO alone ($p=0.002$, Figure 2A, C). The increase in GFP-positive embryos correlated with a 64% average decrease in embryonic viability, consistent with autosome missegregation (Figure 2B) (Hodgkin 2005).

For qualification of the assay, we next tested a set of reference compounds, chemotherapeutic agents, of well-defined aneugenicity. These chemicals have been used extensively in *in vitro* and *in vivo* tests to determine their aneuploidy-inducing potential in mammalian settings. The mode of action and published data describing their mammalian aneugenicity is presented in Supplemental Material, Table S1. We found that known aneugenic agents (bortezomib, dactinomycin, methotrexate, nocodazole, triehtylenemelamine, topotecan, vinblastine sulfate and vincristine) were statistically significant inducers of GFP-positive embryos when compared to DMSO at both 24hr and 65hr time points. We observed that over the combined time points, 7/8 aneugenic compounds were statistical hits, with microtubule drugs (nocodazole, vinblastine sulfate and vincristine) showing the strongest levels of induction. Conversely, all four non-aneugenic compounds tested (5-iodotubercidin, AG1478, Allopurinol and Tyr47) were not different from controls (Figure 2C). The one false negative, thioguanine, may have been missed due to the weak germline *e*xpression in *C. elegans* of hypoxanthine phosphoribosyltransferase 1 (HPRT1), enzyme important for the metabolism and toxicity of thioguanine (Kohara and Shin-i

2013). Finally, bortezomib was toxic at the 65hr time point but positive at 24hr. All together, these results indicate that the *Pxol-1::*GFP reporter strain can be used in a chemical screening setting to accurately discriminate compound aneugenicity.

Screening of environmental compounds with defined mammalian reproductive toxicity

We hypothesized that aneugenic compounds disrupting germline chromosome segregation would likely cause reproductive impairment in mammals. Hence, aneugenic chemicals should be over-represented among those whose exposure leads to decreased fertility and under-represented among those showing no reproductive toxicity. To test this hypothesis, we mined the Toxicological Reference Database (ToxRefDB) from the U.S. Environmental Protection Agency (EPA). This extensive resource compiles over 30 years of mammalian *in vivo* toxicity data on 474 chemicals, primarily pesticides and antimicrobials, and comprises several thousands of *in vivo* endpoints from chronic/subchronic carcinogenicity, prenatal developmental toxicity, and multigenerational reproductive toxicity studies (Knudsen et al. 2009; Martin et al. 2009). The majority of the chemicals in the ToxRefDB, and all of those in the present study, also have associated *in vitro* HTS data in the EPA's ToxCast program across hundreds of human gene and protein targets (Kavlock et al. 2012).

We tested the utility of the meiotic screen by comparing results from a panel of 47 compounds with selected mammalian reproductive endpoints in ToxRefDB that were indicative of decreased female fertility. These *in vivo* endpoints included decreased implantation sites, litter size, early post-natal pup survival, overall reproductive success, reproductive performance, fertility and ovarian morphology defects. The selected compounds were grouped into three categories according to the number of mammalian endpoints they were positive for: high reproductive

toxicity (≥2 endpoints), intermediate reproductive toxicity (1 endpoint) and no reproductive toxicity (0 endpoints). The chemicals tested, their ranking by fold induction in the *C. elegans* assay and their corresponding mammalian *in vivo* endpoint data are presented in Supplemental Material, Table S2 and Table S3. As shown in Figure 3, at 65hr, there is a statistically significant partitioning of all reproductive toxicants (high and intermediate) from compounds that are not ($p=0.008$; two-tailed Mann-Whitney test, 95% C.I.). The 24hr exposure showed a trend toward significance ($p=0.08$). These results indicate a clear enrichment of reproductive toxicants as positive hits from the screen, suggesting that chemical aneugenicity is a likely source of reproductive toxicity in mammals.

Predicting mammalian reproductive impairment from the *C. elegans* screen

We next assessed the predictive value of the *C. elegans* screen with respect to mammalian *in vivo* reproductive toxicity data, where compounds with a lowest effect level in a reproductive study over multiple generations (MG-LEL) ≤500 mg/kg/day were considered positive reproductive toxicants, while those with multigenerational study data but no multigenerational LEL in that range were considered negatives. This cutoff value approximates the reproductive test guideline testing limit of 1000 mg/kg/day and accounts for the large uncertainty around dose measurements and standard conversions applied across many studies and over 30 years of toxicity testing. There was a subset comprised of 7 compounds that did not have associated ToxRefDB data; these were excluded from this portion of the analysis. The data (log fold ratio over DMSO control) from both the 24hr and 65hr exposure intervals were used to predict mammalian reproductive toxicity. As shown in Supplemental Material, Figure S1A and B, we calculated the maximum Balanced Accuracy, which corresponds to the average of sensitivity (ability to correctly identify true positives) and specificity (ability to correctly identify true

negatives) and is therefore a representation of the predictive value of the screen. The balanced accuracy was 68% for the 24hr exposure at a cutoff of 1.6, and 69% for the 65hr exposure at a cutoff of 1.7. Interestingly, at these cutoff values the 24hr exposure provided greater sensitivity (70%) while the 65hr exposure provided greater specificity (78%). For the seven compounds without associated ToxRefDB guideline multigenerational study information, these cutoff criteria identified three positives at both time points (Dimethomorph, Niclosamide and Fenitrothion), two positives at 24hr only (Clorophene and HPTE), and one positive (Methoxychlor) at 65hr only. One compound, Prochloraz, was negative at both time points (Supplemental Material, Table S2, Table S3).

We then calculated the relative risk and associated confidence intervals for each mammalian endpoint indicative of decreased female fertility by iteratively varying the cutoff for a positive result in the *C. elegans* assay, from the lowest observed value to the highest, at each time point. The maximum relative risks for each endpoint, corresponding to a *C. elegans* assay cutoff between 1 and 2 (log fold ratio over DMSO control), are shown in Table 1. In certain cases, higher cutoff values produced larger relative risks, but at the expense of large numbers of false negatives and extreme confidence intervals; therefore we have reported the maximum relative risks corresponding to a cutoff range that optimized the predictive value of the assay. A cutoff of 1.71 at the 65hr time point produced the highest relative risk score (9.69) for the multigenerational rat endpoint of ovary microscopic and gross pathologies and weight changes (termed MGR_Rat_Ovary). While the 65hr time point was most predictive overall for any multigenerational endpoint, the remainder of the endpoints had maximal relative risk scores ranging from 2.56 to 9.69 that were associated with cutoff values of 1.43-1.8 at the 24hr time point. Supporting the strong bias towards predicting reproductive impairment, the screen is not

predictive of other unrelated endpoints such as mammalian liver genotoxicity (Supplemental Material, Figure S2). Together, the results show that the *C. elegans* screening strategy is predictive of mammalian reproductive toxicity with a balanced accuracy approaching 70% and significantly increased relative risk values for reproductive impairment endpoints.

Analysis of meiotic defects from selected compounds

A critical aspect of the screen is the follow-up analysis, albeit not high-throughput, of the chemical hits to discriminate between germline versus early embryonic defects as the source of aneuploidy. To this end, we first monitored the activation of the late pachytene meiotic checkpoint that leads to apoptotic clearing of nuclei carrying unrepaired DNA damage (Gartner et al. 2008). Here, we made use of a strain carrying the *Plim-7ced-1::gfp* transgene to specifically mark engulfed nuclei undergoing apoptosis (Zhou et al. 2001), and compared the 10 most aneugenic compounds (based on fold-change at 65hr) with the 10 least aneugenic ones (Figure 4A). The difference in apoptotic levels between the two groups was extremely significant ($p < 0.0001$ by the two-tailed Mann-Whitney test, C.I. 95%). The statistical comparisons with vehicle control (DMSO) are shown in Figure 4A. The baseline of apoptotic levels were slightly elevated compared to DMSO and a stringent statistical cut-off of $p=0.0001$ of comparison to DMSO was necessary to separate the most and least aneugenic chemicals. This test, however, clearly indicated a dramatic induction of germline apoptosis in many of the top hits from the screen.

Next, we confirmed the presence of meiotic defects in the groups with high levels of apoptosis. Specifically, we observed severe germline defects following exposure to the aneugenic compounds that also induced germline apoptosis (Figure 4B and Supplemental Material, Table

S4). For example, worms exposed to the fungicide Maneb showed severe germline disorganization including gaps or areas with a reduced density of nuclei (Figure 4B; asterisk), which may be due to either impaired meiotic progression or the degeneration of a fraction of nuclei, and a disorganization in the spatial/temporal gradient of meiotic stages (Figure 4B; evidence of intermixing of nuclei at different meiotic prophase stages; red arrowheads). At the stage of diakinesis (end of Prophase I), when fully cellularized oocytes are positioned in a single continuous row in wild type, we also detected unevenly spaced nuclei suggesting a defect in cytokinesis (white arrows). Interestingly, both gaps and intermixing of nuclei at different meiotic stages (red arrows) were also observed following exposure to the fungicide TCMTB. None of these defects was observed in the DMSO exposed control worms or in animals exposed to other compounds (Supplemental Material, Table S4). Together, these experiments strongly suggest a meiotic origin for the embryonic aneuploidy detected in the screen. This strategy therefore provides a fast and reliable tool to elucidate environmental influences on germline function and predict mammalian reproductive toxicity.

High-throughput adaptation and chemical sensitization

Finally, we propose a technology that can be readily applied in a HTS assay. We used an automated fluorescence assisted sorter for large objects (COPAS BIOSORT, Union Biometrica) that can accurately read and sort whole animals as well as embryos from a suspension of worm culture (Boyd et al. 2010a; Boyd et al. 2010b). To verify that the worm sorter can detect the presence of aneuploidy/GFP-positive embryos, we first sorted two genetically distinct worm populations: *Pxol-1::gfp* and *Pxol-1::gfp; him-8(e1489)* worms. HIM-8 is a protein that associates with a region known as the pairing center on the X-chromosome in *C. elegans* whose activity is essential for the proper segregation of the X-chromosome during meiosis (Hodgkin et

al. 1979). Thus, *him-8(e1489)* mutants produce a high number of male progeny (approximately 30%) due to increased X chromosome missegregation that can be easily visualized in the context of the *Pxol-1::gfp; him-8(e1489)* strain (Figure 5A). Automated reading of the two populations easily identified a clear subset of GFP-positive embryos that were present in much lower numbers in the *Pxol-1::gfp* worms alone, which allowed us to determine a threshold to discriminate between GFP-positive and GFP-negative embryos and any remnants of culture debris.

Next, we tested the sorting of worms exposed to nocodazole and compared it to DMSO. Automated reading readily identified two distinct groups based on fluorescence levels, with approximately 3-fold induction between nocodazole-treated and control worms (Figure 5B). We also chemically sensitized the *Pxol-1::gfp* strain by incorporating the mutant allele *nx3* of the cuticle collagen gene *col-121* which was isolated in a screen for hypersensitivity to bisphenol A (Watanabe et al. 2005). In this background, a 2.7 fold increase in GFP-positive embryos was observed in DMSO treated worms compared to *Pxol-1::gfp* alone, possibly due to increased sensitivity to the low aneugenic activity of DMSO (Goldstein and Magnano 1988). However, the number of GFP-positive events captured was also dramatically improved: about 220% more for the same number of worms sorted. Thus, automated detection and sorting of the worms is a valuable option for the HTS screening of chemical aneuploidy.

Discussion

The results from this study demonstrate an efficient and reliable technology for the fast screening of chemicals altering germline function. We have focused on environmental compounds as a mean to address a gap in our current ability to assess the potential hazards of thousands of

untested chemicals. However, the assay described here is also applicable to other chemical screens including drug safety assessment and small molecule assays for the analysis of germline pathways.

We estimate that the screening time with the COPAS BIOSORT will consist of 65 hours of exposure followed by 45 minutes of reading for each 96-well plate. As exposures can be performed simultaneously and each plate only adds an additional 45 minutes of reading time, a library of 1,000 compounds could be screened in triplicate in a minimum period of 4 days. The running costs of the screen are extremely low as the only reagents necessary for the screen are deep 96-well plates, buffer solution and bacteria for food. By comparison, mammalian reproduction assays are much costlier and lengthier. A typical single generation rodent reproduction test involves an 8 to 10 week exposure window starting around puberty and comprising a minimum of 20 pregnancies for each dose group (U.S. Environmental Protection Agency 1996). Mammalian cell based assays, on the other hand, do not recapitulate efficiently all stage of meiosis and are not suitable for large scale platforms. Thus, we are providing a unique whole organism first tier assay which examines the outcome of complex cellular and developmental processes with short running time, modest cost and high accuracy.

The “green eggs and Him” output is representative of overall levels of aneuploidy as evidenced by: (1) the correlation between GFP expression and the presence of germline defects as well as high levels of embryonic lethality, a phenotype expected from the missegregation of the autosomes and not just the X-chromosome (Hodgkin 2005; Kelly et al. 2000); (2) the fact that most genetic disruption of the germline lead to missegregation of all chromosomes and not just the X-chromosome (Dernburg et al. 1998; Hodgkin et al. 1979; Kelly et al. 2000); and (3) that exposures to at least two hits from the screen: Maneb and TCMTB show a high level of

germline disruption indicating that the GFP readout utilized can indicate the disruption of germline processes. Nonetheless, although not performed in this study, a follow-up analysis of selected hits should include the measurement of embryonic lethality. This measurement requires significant time and cannot be embedded within an HTS assay. However, it will permit further validation of the hits as affecting other chromosomes alongside the X-chromosome.

An interesting feature of the screening results is that some of the strong aneuploidy-inducing hits (Supplemental Material, Table S2, Table S3) lack any described mammalian reprotoxicity. Although we cannot explain the presence of all of these compounds near the top of the list, some of them have well described mammalian germ cell aneugenicity such as thiabendazole (ranked 8th at 65hr; Supplemental Material, Table S3) (Mailhes et al. 1997; Schmid et al. 1999). The outcome of our screen, together with past aneugenic evidence, predicts a potential reproductive hazard of thiabendazole. Finally, some compounds are negative hits in the screen, implying that they produce less aneuploidy than DMSO alone. A possible explanation for this would be the undesirable direct inhibition of reporter expression. However, the number of chemicals exerting such an effect is low (2/47 at 24hr and 65hr) and is manageable in the context of a first pass screening strategy.

A difference in ADME parameters, such as for thioguanine, will be a likely source of false negatives in *C. elegans* compared to mammalian systems. Furthermore, a potential avenue for improving the screen could be the inclusion of dose-response curves for each compound. However, a distinct advantage to the present approach is the ability to screen with varying sensitivity or specificity depending on the application, be it risk assessment or the identification of mechanism of action. The cutoff criteria for a positive hit in the *C. elegans* assay may be optimized accordingly, where a value of 1.2 at the 24hr exposure provides a sensitivity of 80%,

and a value of 2.3 in the 65hr exposure results in a specificity of 89%. Using this method removes many of the aforementioned false negatives or false positives, respectively. As shown in Table 1, higher relative risks for reproductive endpoints such as offspring viability and litter size corresponded to lower cutoff values at the 24hr time point, and higher relative risk for endpoints such as fertility and ovarian pathology corresponded to higher cutoff values or the later 65hr time point. The differing time points and cutoff values may provide information on varying events in the meiotic process, embryonic stages, or specific reproductive organs that may be targeted or impaired by different chemicals.

Conclusion

With a low cost, high speed and strong predictive value, this technology fulfills the requirement for first pass assessment of chemical hazard, and furthermore offers insight into germline disruption as a mechanism of reproductive toxicity.

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Table 1: Relative risk[a]

Reproductive Toxicity Endpoint	Relative Risk	95% Confidence Intervals	(+)Cutoff: *C. elegans* assay	Time Point
MGR_Rat_Fertility	4.05	(0.35,46.60)	1.80	24hr
MGR_Rat_LitterSize	6.82	(0.82,56.76)	1.64	24hr
MGR_Rat_Ovary	9.69	(1.11,84.53)	1.71	65hr
MGR_rat_ReproductiveOutcome	8.08	(1.11,58.93)	1.43	24hr
MGR_rat_ReproductivePerformance	9.45	(1.19,74.85)	1.80	24hr
MGR_Rat_ViabilityPND4	2.56	(1.49,2.39)	1.64	24hr
mgLEL (any)	2.15	(1.75,2.64)	1.69	65hr

[a]For each reproductive toxicity endpoint, the relative risk was calculated by iteratively increasing the cutoff value (log fold ratio over DMSO control) for a positive result in the *C. elegans* assay at each time point. Maximum relative risks and confidence intervals are shown for cutoff values within a range shown to maximize the predictive value of the assay.

Abbreviations: MGR: multigenerational, PND4: Post Natal Day 4

Figure Legends

Figure 1. Design of the screening platform. Worms from the aneuploidy-reporting *Pxol-1::GFP* strain are exposed to libraries of environmental compounds for either 24hrs or 65hrs. Following exposure, the induction of aneuploidy can be visualized and quantified by fluorescence microscopy (bottom left, bar=100µm) or automated detection and sorting of the worms (bottom right). In left panel, several embryos expressing GFP (GFP+) can clearly be visualized. The right panel shows automated reading of the embryos. X-axis: time of flight, Y-axis: GFP peak height. A population of GFP+ embryos can be detected as distinct from GFP- embryos and debris found below the black bar.

Figure 2. Chemical induction of aneuploidy in *C. elegans.* A. *Pxol-1::GFP* worms were exposed to 100µM nocodazole or 0.1% DMSO for 24hr. Two GFP+ embryos are visible within the nocodazole treated worm's uterus (red arrows) adjacent to the autofluorescence emanating from the gut. Bar=50µm. B. Embryonic viability following either DMSO or nocodazole exposure. The asterisk indicates statistical significance ($p \leq 0.05$, two-tailed Mann-Whitney test, 95% C.I.). Error bars=SEM. C. Chemotherapeutic screen. The worms were exposed for 24hr (dashed blue or red) or 65hr (solid blue or red) to 100µM of each compound. Blue=non-aneugenic compounds, red=aneugenic compounds. The number of GFP+ embryos per worm was recorded, corrected for the average number of embryos found in each worm and expressed as the log fold ratio over DMSO. Because of high differences in sample variance, a two-tailed Mann-Whitney test, 95% C.I. was chosen over ANOVA with post-test correction to test for significant differences for each compound over DMSO (*$p \leq 0.05$; **$p \leq 0.01$). ND=Bortezomib was lethal at 65hr. Error bars=SEM, each chemical exposure performed six times.

Figure 3. Screening of environmental chemicals. Worms were exposed for either 24h (A) or 65h (B) to each compound, at a concentration of 100µM (unless specified otherwise in the material and methods section). The number of green embryos per worm was recorded and corrected for the average number of embryos found in each worm. The number was then expressed as the log fold ratio over DMSO. Chemical names can be found in Supplemental Material, Table S2 and Table S3 ranked in the same order. The compounds were categorized according to their assessed mammalian reproductive toxicity, i.e. the number of mammalian endpoints that they were positive for: high reproductive toxicity (>2 endpoints, dark red), intermediate reproductive toxicity (1 endpoint, dashed red) and no reproductive toxicity (0 endpoints, light blue). The black bar represents nocodazole (positive control). At 65hr, the mean value of fold-induction for the reprotoxic groups was significantly higher than the non-reprotoxic group ($p=0.008$). Error bars=SEM, each chemical was tested in triplicate.

Figure 4. Functional validation of selected compounds. A. Germline apoptosis assay quantification. Following exposure to each of the 10 least aneugenic compounds in the *C. elegans* assay (left, blue) or to each of the 10 most aneugenic compounds (right, red), we quantified apoptotic levels through the use of the *Plim-7ced-1::gfp* reporter. Asterisks represent the statistical significance of 9 compounds over DMSO (*$p<0.001$, ANOVA test followed by Dunn's comparison). Also, the numbers of apoptotic nuclei in the 10 least and most aneugenic compounds were highly significantly different from each other ($p<0.0001$; two-tailed Mann-Whitney test, 95% C.I.). DMSO was used as a negative control and the DNA damaging agent camptothecin as a positive control. The lower and upper edges of the box plot represent the 1st and 3rd quartiles respectively, with the median represented as a line within the box. The whiskers extend to +/-1.5xinterquartile range (IQR). B. DAPI staining of germline nuclei revealed

profound germline defects following exposure to Maneb and TCMTB. Shown here are assembled germlines showing areas of reduced nuclear density (asterisk), intermixed meiotic stages (red arrowheads) and unequally spaced diakinetic nuclei (white arrows). Insets show high magnification examples of late diakinetic nuclei. Note that nuclei with chromosomes in a pachytene stage-like organization are present intermixed with diakinetic nuclei in late prophase following Maneb exposure as shown in inset. Bar=50µm; bar within inset=4µm.

Figure 5. High-throughput detection of genetic and chemical induction of aneuploidy. A. Overlay of bright field and fluorescence images of a *Pxol-1::GFP, him-8* worm with two embryos. The GFP-positive embryo (red arrow) is clearly distinguishable from the GFP-negative embryo next to it. Bar=100µm. B. Automated detection of GFP-positive embryos using the COPAS Biosort. The black line represents the GFP-positive threshold as determined by *Pxol-1::GFP* without *him-8* (left most panel). The GFP-positive population of embryos is more abundant in nocodazole treated worms compared to DMSO treated in both the *Pxol-1::GFP* and the *Pxol-1::GFP; col-121* (sensitized) backgrounds.

Figure 1

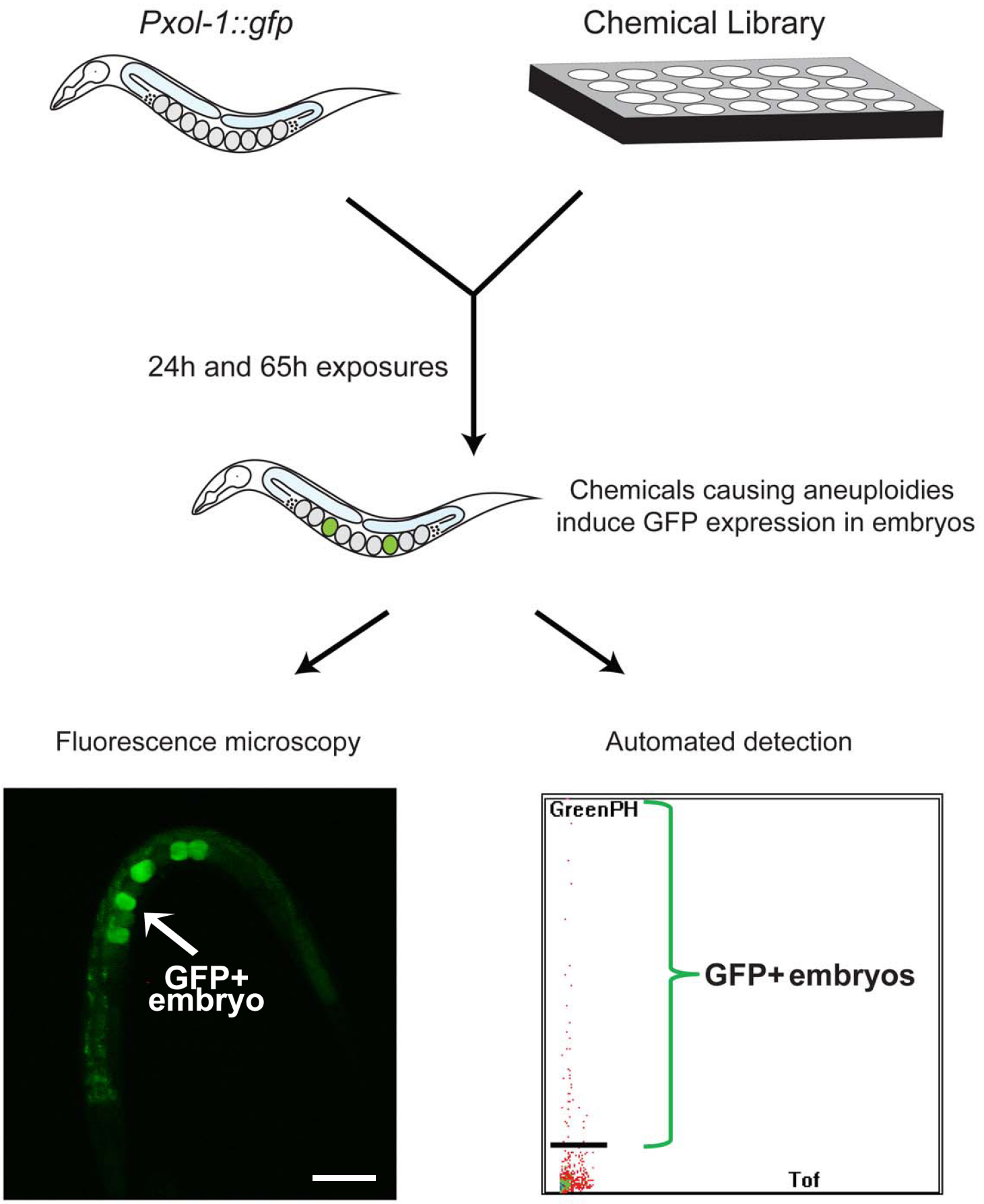

Pxol-1::gfp
Chemical Library
24h and 65h exposures
Chemicals causing aneuploidies
induce GFP expression in embryos
Fluorescence microscopy
Automated detection
GFP+
embryo
GreenPH
GFP+ embryos
Tof

Figure 2

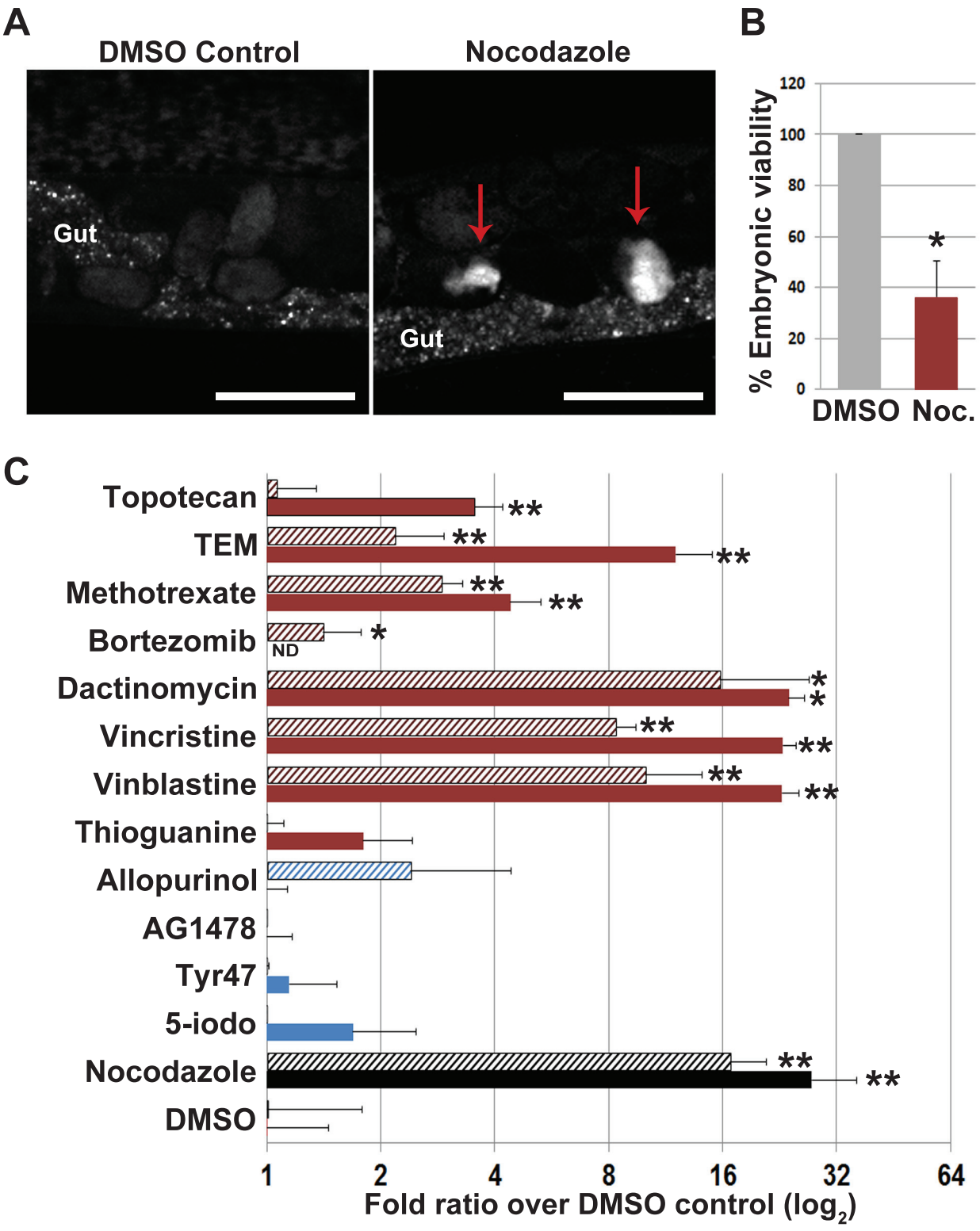

A
DMSO Control
Nocodazole
Gut
Gut
B
% Embryonic viability
120
100
80
60
40
20
0
*
DMSO
Noc.
C
Topotecan
TEM
Methotrexate
Bortezomib
ND
Dactinomycin
Vincristine
Vinblastine
Thioguanine
Allopurinol
AG1478
Tyr47
5-iodo
Nocodazole
DMSO
**
*
1
2
4
8
16
32
64
Fold ratio over DMSO control (log2)

Figure 3

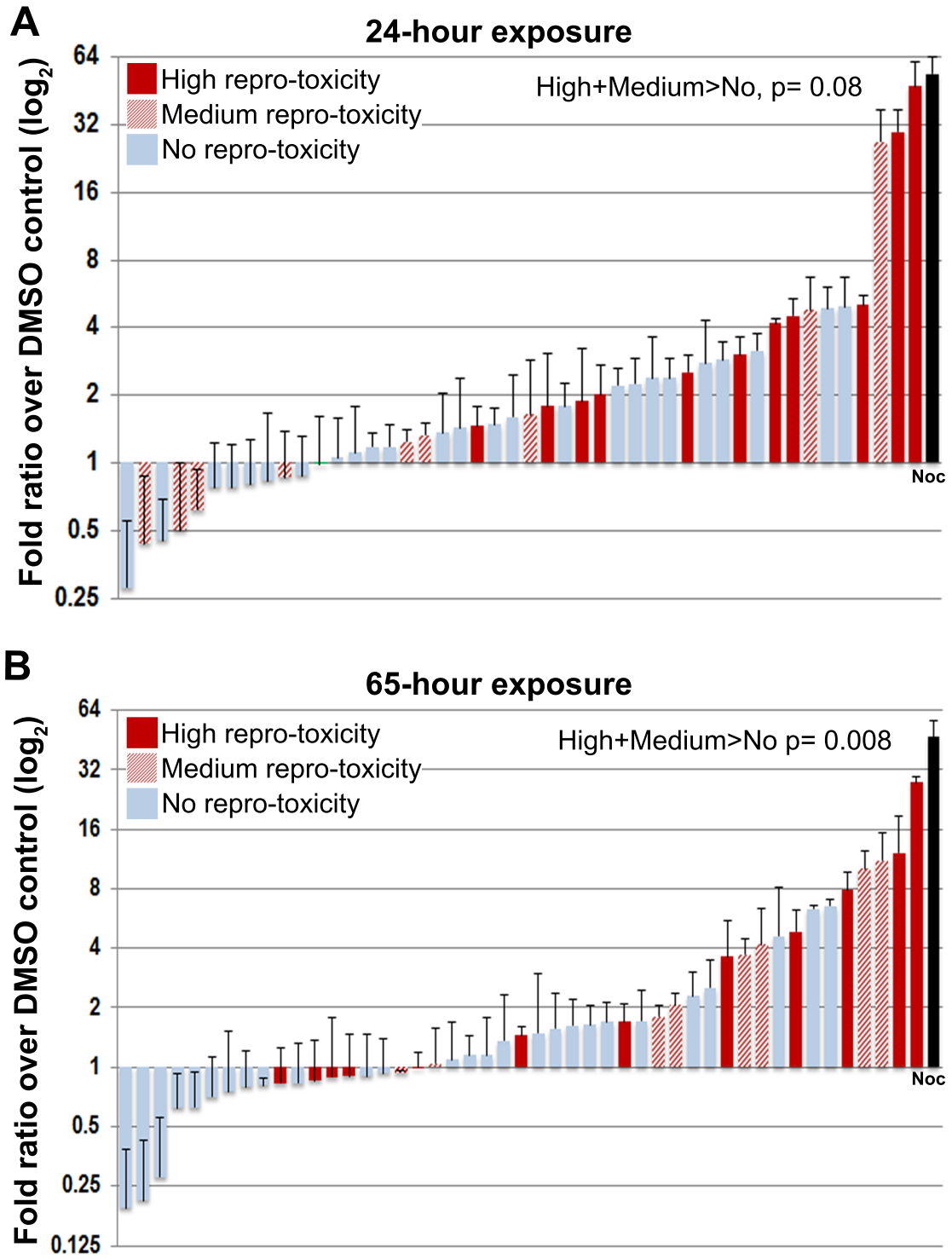

A
24-hour exposure
High repro-toxicity
Medium repro-toxicity
No repro-toxicity
High+Medium>No, p= 0.08
Fold ratio over DMSO control (log2)
64
32
16
8
4
2
1
0.5
0.25
Noc
B
65-hour exposure
High repro-toxicity
Medium repro-toxicity
No repro-toxicity
High+Medium>No p= 0.008
Fold ratio over DMSO control (log2)
64
32
16
8
4
2
1
0.5
0.25
0.125
Noc

Figure 4

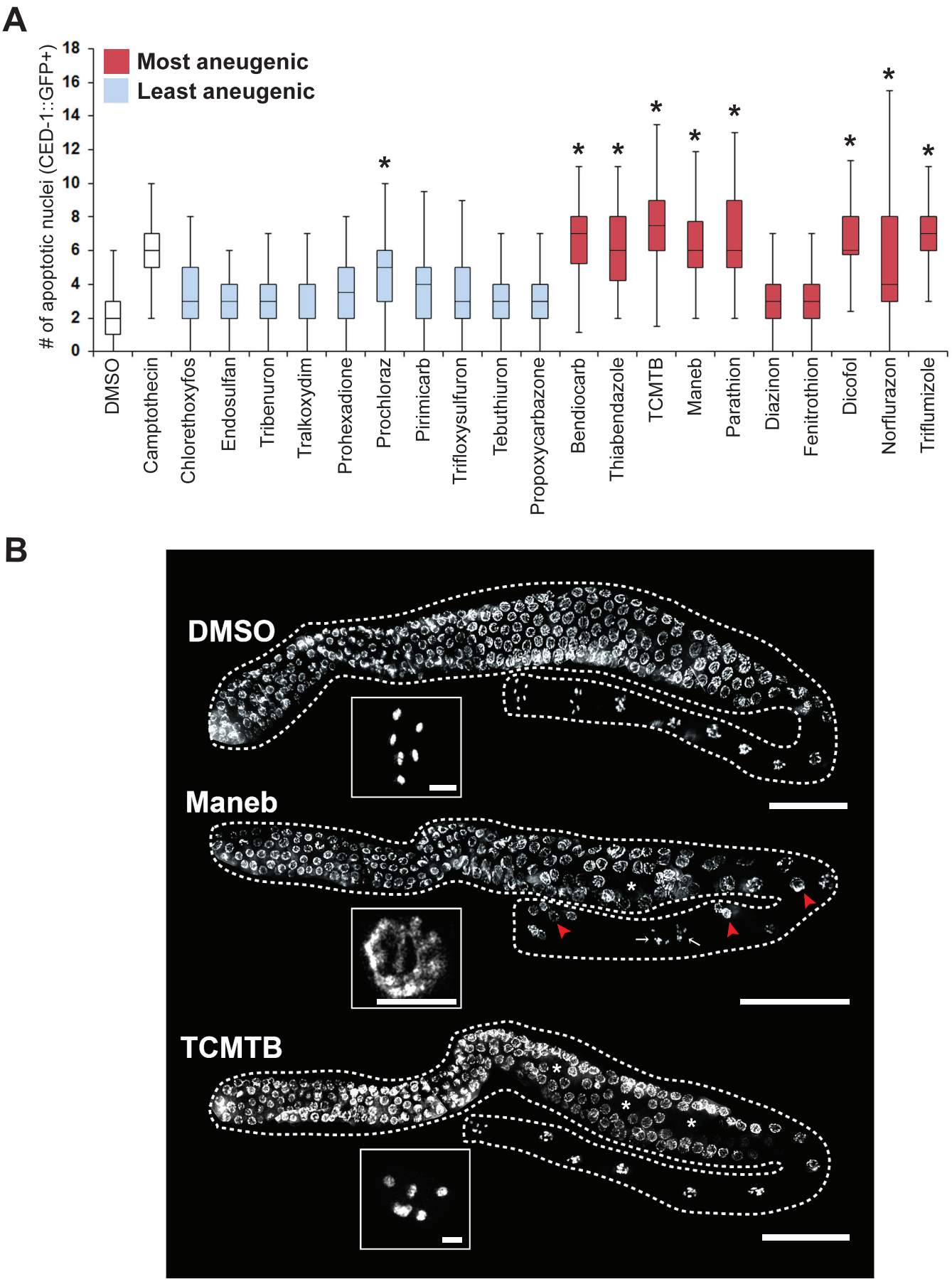
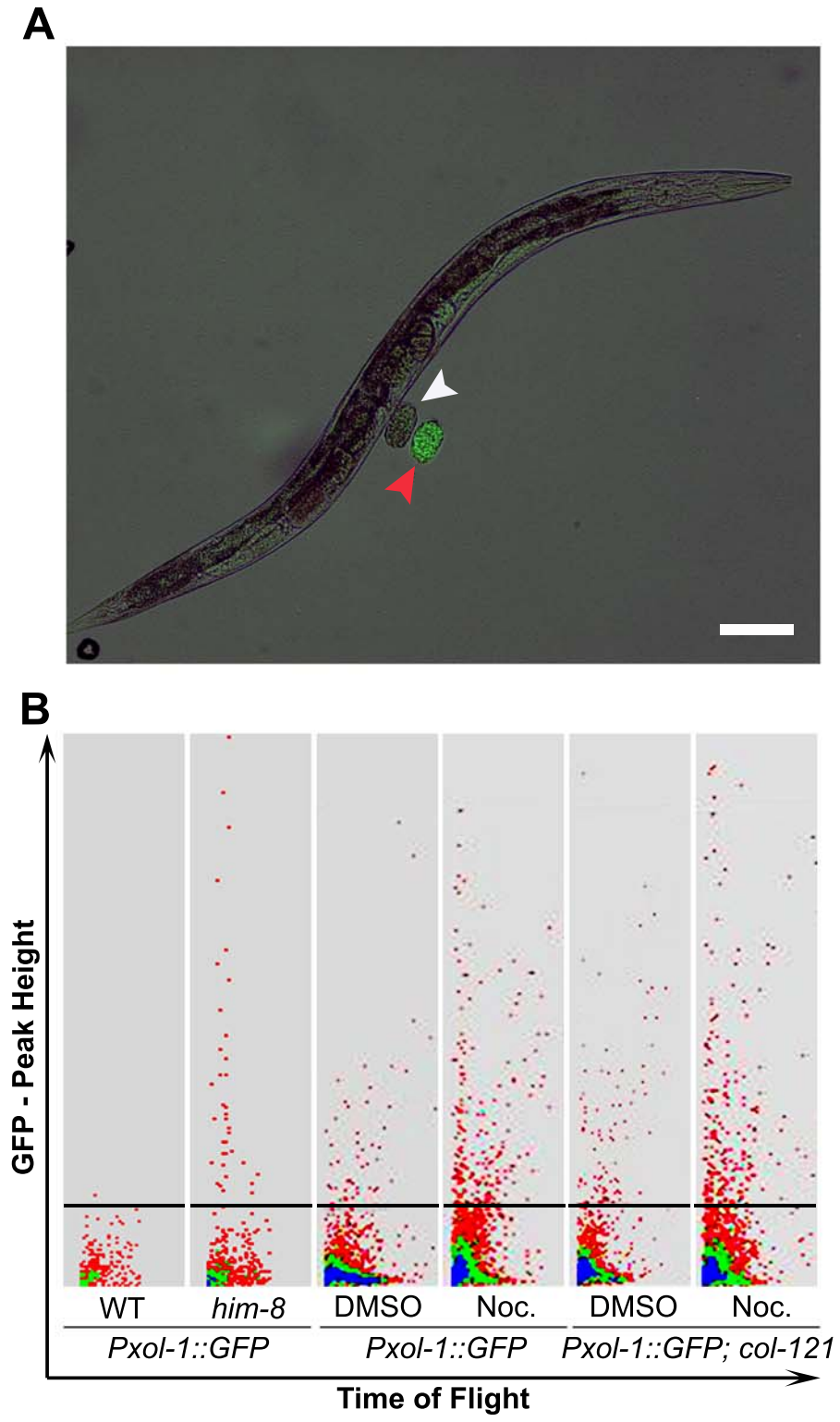

A
Most aneugenic
Least aneugenic
# of apoptotic nuclei (CED-1::GFP+)
0
2
4
6
8
10
12
14
16
18
DMSO
Camptothecin
Chlorethoxyfos
Endosulfan
Tribenuron
Tralkoxydim
Prohexadione
Prochloraz
Pirimicarb
Trifloxysulfuron
Tebuthiuron
Propoxycarbazone
Bendiocarb
Thiabendazole
TCMTB
Maneb
Parathion
Diazinon
Fenitrothion
Dicofol
Norflurazon
Triflumizole
B
DMSO
Maneb
TCMTB

Figure 5


A
B
GFP - Peak Height
WT
him-8
DMSO
Noc.
DMSO
Noc.
Pxol-1::GFP
Pxol-1::GFP
Pxol-1::GFP; col-121
Time of Flight